

Accepted Manuscript

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PII: S0091-6749(18)30030-7

DOI: [10.1016/j.jaci.2017.10.048](https://doi.org/10.1016/j.jaci.2017.10.048)

Reference: YMAI 13209

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 9 June 2017

Revised Date: 7 September 2017

Accepted Date: 17 October 2017

Please cite this article as: Lind C, Skaarup SH, Lorentz A, Hoffmann HJ, Basophil testing with CD63 in pollen sensitised patients is independent of the circadian clock, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2017.10.048.

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Basophil testing with CD63 in pollen sensitised patients is independent of the circadian clock

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21 **Capsule summary**

22 Basophil activation, a promising marker of allergy, may be controlled by the circadian clock. We
23 tested response to allergen through CD63 and CD203c over a 24-hour period. CD63 expression is
24 not subject to diurnal variation.

25

26

27 **Key Words**

- 28 • Basophil Activation
- 29 • BAT
- 30 • CD63
- 31 • CD203c
- 32 • Diurnal variation
- 33 • Circadian clock
- 34 • Allergy

35

36

37 **Abbreviations**

38 BAT Basophil activation test

39 CD-sens 100 x the inverse of EC50

40 EC50: the allergen concentration leading to half maximal activation of basophils

41 MFI Mean Fluorescence Intensity

42

43

44 *To the Editor:*

45 Allergy is a public health issue that affects more than 25 % of western populations. Diurnal
46 variation of allergic symptoms is well documented; exacerbations frequently occur at night and in
47 the early morning, and may be associated with a diurnal lowering of the threshold for histamine
48 sensitivity in the bronchial tree¹. The pathomechanism underlying these observations is not
49 understood, but is thought to involve mast cell mediators. Diurnal variation of clock genes and
50 release of histamine and synthesis of cysteinyl leukotrienes were measured from human intestinal
51 mast cells synchronised with dexamethasone². Basophil activation testing (BAT) is a useful second
52 line test for allergy, that documents crosslinking of IgE by allergen³ and can be used to document
53 and monitor severity of allergy. Basophil granulocytes are short-lived blood leukocytes, whereas
54 mast cells mature, and live in tissue for up to three months. Although basophil sensitivity has been
55 shown to be very reproducible over a two week interval⁴ evidence of diurnal variation on basophil
56 testing would severely limit this useful technique. A recent study identified variation of expression
57 of mRNA for clock genes *Per1* and *Per2* in human basophils, suggesting circadian influence on the
58 IgE mediated basophil response⁵. Functional data suggest that these genes may influence basophil
59 activity; surface expression of the activation marker CD203c was higher at 6:00 than at 18:00⁵. We
60 could not confirm this observation in a pilot study⁶, in which neither reactivity (% CD63+ basophils)
61 nor sensitivity (EC50 or CD-sens) of basophils of non-allergic individuals stimulated with anti-FcεRI
62 antibody were subject to diurnal variation. Here we assessed the presence of diurnal variation in
63 basophil reactivity and sensitivity to activation by clinically relevant allergen.

64

65 We investigated 11 allergic young adults (median age 25,82 years) over a 24-hour period outside
66 the pollen season. Volunteers completed an allergy questionnaire of the Allergy Center at Aarhus
67 University Hospital and were included on clinical symptoms of seasonal rhino conjunctivitis and
68 specific IgE to either grass or birch (n=9 and n=2). For clinical characteristic see table 1. Peakflow,
69 BAT and serum melatonin measurement were performed at six time points (13.00 h, 16.00 h,
70 19.00 h, 1.00 h, 4.00 h, 7.00 h) as previously described⁶. Basophils were identified with CD193
71 Alexa Fluor 647 and activation assessed by incubation at 37C for 30 minutes with the most
72 relevant allergen, based on the patient history and specific IgE, in nine tenfold dilutions starting
73 with 1000 SQU/ml ALK Aquagen and markers CD63-FITC and CD203c-PE (Biolegend, San Diego,

CA). Statistical analyses were performed with Prism 6.0 (GraphPad, California), and the dose/response were fitted to a 4-parameter nonlinear regression model. Maximal activation, basophil reactivity and basophil sensitivity EC50 were calculated. $P > 0,05$ in a one-way ANOVA excluded diurnal variation. In a paired t-test, used to test for differences at 12 hours' interval, $p < 0,05$ was considered significant. Retrospective diurnal allergic symptoms of the volunteers varied from every night to few times a week in 7/11 participants (Table 2). Four of seven reported that the frequency of nocturnal symptoms increased in the pollen season. Peak flow measurements confirmed diurnal variation in lung function ($p = 0,0033$) (Figure 1.A). Serum melatonin levels varied significantly over 24 hours ($p < 0,0001$), confirming a functional circadian clock in the volunteers (Figure 1.B). We found that the fraction of CD63 positive basophils response did not vary over a 24-hour period (Figure 1C), but a significant difference in the fraction of CD203c positive basophils with one-way ANOVA ($p = 0,034$), reflected in an increase in CD203c reactivity at low allergen concentration between 13.00 H and 1.00 H ($p = 0,0431$) consistent with diurnal variation of CD203c expression (Figure 1.D). However, when we compared basophil reactivity and sensitivity for CD63 and CD203c there was no significant variation (Figure 1.E, F). There was no diurnal variation in the resting MFI for CD63 or CD203c on blood basophils (not shown). We previously found a significant difference in mean fluorescence intensity (MFI)⁶; that could not be confirmed in this study with allergic volunteers.

In this study, we found expression of CD63 to be a stable marker for allergen activation of basophil granulocytes that did not vary over the investigated 24-hour period. We did see the expected significant variation in plasma melatonin concentration, PEF and subjective symptoms. The well-known variation in allergic symptoms is thus not reflected in upregulation of CD63 to the cell surface, a common marker for basophil degranulation. We thus strengthen our previous finding, that CD63 expression is not subject to diurnal variation, now with relevant allergen rather than with anti-FcεRI antibody and at six time points to allow us to perform relevant statistical analyses⁶. Upregulation of CD203c on basophils was significant at low allergen concentration as well as in analysis of diurnal variation, but did not achieve statistical significance in basophil reactivity and sensitivity. Both CD63 and CD203c are validated as a marker for basophil activation³. Where CD63 is located in the secretory lysozymes containing preformed mediators like histamine and is truly

104 associated with anaphylactic degranulation of blood basophils, CD203c is found in small vesicles⁷.
105 Our and others⁴ data suggest that there is little variation in BAT through CD63. Ando et al⁵ and we
106 show that CD203c may be subject to diurnal variation, but an association with circadian rhythm
107 must still be ascertained. Other explanations for diurnal variation of allergic symptoms may be
108 sleeping posture, environmental factors⁸ or a circadian tissue response¹. Mast cells express clock
109 genes² and IgE mediated release of tryptase from dexamethasone synchronised mast cells in vitro
110 displays diurnal variation², indicating that the mast cell response could contribute to diurnal
111 allergic symptoms. However, diurnal variation in skin reactivity to allergen and histamine was
112 similar⁹, suggesting that the variation resides in the tissue response to histamine rather than in
113 mast cell reactivity⁹. We see little evidence for a role of the basophil granulocyte in this
114 mechanism. In conclusion, CD63 is a reliable marker for ex vivo allergen activation of basophil
115 granulocytes. It was stable during a 24-hour period and is therefore the first choice of basophil
116 activation marker.

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Legends

Figure 1: A, Normalized PEF to the median for each individual (median and IQR) ($p=0,0033$). B, Serum Melatonin (mean $\pm$ SD) over time which showed an expected pattern, with high levels at night compared to daylight levels ($p>0,0001$). C-D, Dose/response curves for CD63 and CD203c pictured as mean $\pm$ SD. The curves are paired with 12-hour interval, to visualize the difference over time. Only CD203c activation changes significant over time (one-wayANOVA ($p=0,0340$) and a significant change with paired t test between 13.00H and 1

Table 1: Clinical Characteristics: Seminal clinical characteristics of patients enrolled in this study are presented as either fraction of patients presenting with a condition, or median of a metric.

Table 1 Clinical characteristic	
Gender, female/male	8/3
Age in years (median, range)	25,82 (20-46)
Duration of allergy (median years)	17,64
Atopy in family	8/11
Mono-sensitized	1/11
Grass specific (g6) sIgE (median KU _A /l) N=9	47,31
Birch specific (t3) sIgE (median KU _A /l) N=2	1,65
Other sensitizations (N)	
House dust mite (d1)	7/11
Birch (t3)	5/11
Mugwort (w6)	8/11
Dog (e5)	7/11
Cat (e1)	6/11

161 Table 2: Self-reported frequency of nocturnal symptoms ("do you have night time symptoms from
 162 the lung or nose"): – less than two times a month, *less than one time a week, ** more than one
 163 time a week, *** every night

Subject #	Jan.	Feb.	Mar.	Apr.	May	June	July	Sep.	Oct.	Nov.	Dec.
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-
3	**	**	**	**	**	**	**	**	**	**	**
4	-	-	-	-	***	***	***	***	-	-	-
5	**	**	**	**	**	**	**	**	**	**	**
6	***	***	**	**	**	***	***	***	***	***	***
7	***	***	***	***	***	***	***	***	***	***	***
8	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	**	**	**	**	**	-	-	-
11	-	-	*	*	-	-	-	*	*	-	-

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